

University of Groningen

Cysteine cross-linking defines part of the dimer and B/C domain interface of the Escherichia coli mannitol permease

van Montfort, B.A.; Schuurman-Wolters, G.K.; Duurkens, R.H.T.; Mensen, R.; Poolman, B.; Robillard, G.T.

Published in:

The Journal of Biological Chemistry

DOI:

[10.1074/jbc.M010728200](https://doi.org/10.1074/jbc.M010728200)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2001

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

van Montfort, B. A., Schuurman-Wolters, G. K., Duurkens, R. H. T., Mensen, R., Poolman, B., & Robillard, G. T. (2001). Cysteine cross-linking defines part of the dimer and B/C domain interface of the Escherichia coli mannitol permease. *The Journal of Biological Chemistry*, 276(16), 12756 - 12763.
<https://doi.org/10.1074/jbc.M010728200>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Cysteine Cross-linking Defines Part of the Dimer and B/C Domain Interface of the *Escherichia coli* Mannitol Permease*

Received for publication, November 28, 2000, and in revised form, January 11, 2001
Published, JBC Papers in Press, January 17, 2001, DOI 10.1074/jbc.M010728200

Bart A. van Montfort, Gea K. Schuurman-Wolters, Ria H. Duurkens, Robert Mensen,
Bert Poolman, and George T. Robillard‡

From the Groningen Biomolecular Sciences and Biotechnology Institute and the Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Part of the dimer and B/C domain interface of the *Escherichia coli* mannitol permease (EII^{mtl}) has been identified by the generation of disulfide bridges in a single-cysteine EII^{mtl}, with only the activity linked Cys³⁸⁴ in the B domain, and in a double-cysteine EII^{mtl} with cysteines at positions 384 and 124 in the first cytoplasmic loop of the C domain. The disulfide bridges were formed in the enzyme in inside-out membrane vesicles and in the purified enzyme by oxidation with Cu(II)-(1,10-phenanthroline)₃, and they were visualized by SDS-polyacrylamide gel electrophoresis. Discrimination between possible disulfide bridges in the dimeric double-cysteine EII^{mtl} was done by partial digestion of the protein and the formation of heterodimers, in which the cysteines were located either on different subunits or on one subunit. The disulfide bridges that were identified are an intersubunit Cys³⁸⁴-Cys³⁸⁴, an intersubunit Cys¹²⁴-Cys¹²⁴, an intersubunit Cys³⁸⁴-Cys¹²⁴, and an intrasubunit Cys³⁸⁴-Cys¹²⁴. The disulfide bridges between the B and C domain were observed with purified enzyme and confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Mannitol did not influence the formation of the disulfide between Cys³⁸⁴ and Cys¹²⁴. The close proximity of the two cysteines 124 was further confirmed with a separate C domain by oxidation with Cu(II)-(1,10-phenanthroline)₃ or by reactions with dimaleimides of different length. The data in combination with other work show that the first cytoplasmic loop around residue 124 is located at the dimer interface and involved in the interaction between the B and C domain.

The uptake and concomitant phosphorylation of a wide variety of carbohydrates into bacterial cells is, in many cases, accomplished by the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (1). In a cascade of phosphorylation reactions (Fig. 1), the phosphoryl group is transferred from the enzyme donor phosphoenolpyruvate (PEP)¹ via the two general

components EI and HPr to the carbohydrate-specific components (Enzyme II's), which transport and phosphorylate the carbohydrates (2). All EII's² have a similar architecture and consist of the cytoplasmic A and B domains and a membrane-embedded C domain. This article deals with the mannitol-specific EII (EII^{mtl}) of *Escherichia coli*, in which the three domains are covalently linked. HPr phosphorylates the A domain of EII^{mtl} on His⁵⁵⁴, which subsequently phosphorylates Cys³⁸⁴ in the B domain. Mannitol in the periplasm is bound by the C domain, transported into the cell via C and, while bound at the cytoplasmic site of C, phosphorylated by the B domain. EII^{mtl} is most likely a dimeric protein and the subunit interactions occur in the C domain (3–8).

Domain interactions and in particular the B/C domain interface play an important role in the catalytic cycle of EII^{mtl}. The energy coupling mechanism involves conformational interaction between the B and C domain. The evidence for this notion is manifold. 1) Phosphorylation of the B domain increases the rate of transport 2–3 orders of magnitude (9, 10). 2) Modification or mutagenesis of the phosphorylation site in the B domain as well as removal of the cytoplasmic domains changes the mannitol binding kinetics of the C domain (11, 12). 3) Time-resolved fluorescence and phosphorescence spectroscopy showed that, upon phosphorylation of the B domain, Trp¹⁰⁹ in the C domain becomes immobilized whereas Trp³⁰ in the C domain becomes more flexible (13, 14). 4) Differential scanning calorimetry showed that the thermal stability of the C domain is higher in the presence of the B domain (15). 5) Isothermal titration calorimetry experiments indicated that a significant part of the structural changes upon the binding of mannitol to the C domain reside in the B domain. Approximately 50–60 residues are removed from the bulk water upon binding of mannitol, which was much less when the same measurements were done after removal of the B domain (16). 6) Close proximity of the B and C domain has been suggested for another PTS transporter, that is the BglF system of *E. coli*.³

To date, there is no structural information about the B/C domain or dimer interface of EII^{mtl} or any other EII. The topological model of the C domain predicts 6 membrane-span-

* This work was supported by the Ministry of Economic Affairs, the Ministry of Education, Culture and Science, and the Ministry of Agriculture, Nature Management and Fishery in the framework of an industrial relevant research program of the Netherlands Association of Biotechnology Centers in the Netherlands (ABON). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Tel.: 31-50-3634321; Fax: 31-50-3634429; E-mail G.T.Robillard@chem.rug.nl.

¹ The abbreviations used are: PEP, phosphoenolpyruvate; ISO, inside-out; dPEG, decyl-polyethylene glycol; CuPhe, Cu(II)-(1,10-phenanthroline)₃; PTS, phosphotransferase system; mtl, mannitol; HPr, histidine-containing protein; EI, enzyme I; MALDI-TOF MS, matrix-

assisted laser desorption-ionization-time-of-flight mass spectrometry; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

² The nomenclature of the enzymes is: EII, carbohydrate-specific transport protein of the PTS; EII^{mtl}, wild-type mannitol-specific EII of *E. coli* containing all four cysteines; SSCS, N-terminal His-tagged EII^{mtl} with Cys¹¹⁰, Cys³²⁰, and Cys⁵⁷¹ replaced with a serine; SSCS-S124C, SSCS with Ser¹²⁴ in the C domain replaced with a cysteine; IIChis-CL, C-terminal His-tagged cysteine-less C domain with Cys¹¹⁰ and Cys³²⁰ replaced with serine; IIChis-S124C, IIChis-CL in which Ser¹²⁴ is replaced with a cysteine. Numbering of residues, even in the His-tagged mutants, is always according to the numbering in the original sequence of EII^{mtl}.

³ O. Amster-Choder, personal communication.

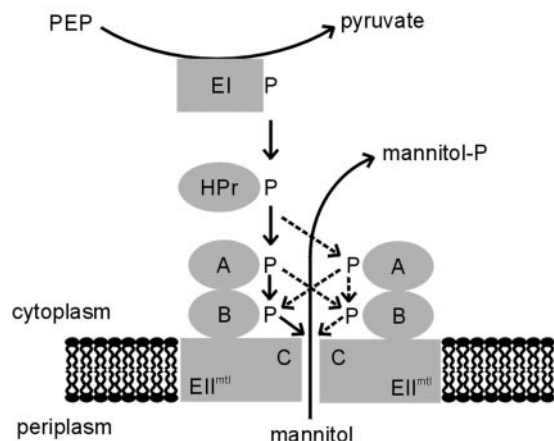


FIG. 1. Schematic representation of the mannitol-specific phosphoenolpyruvate-dependent phosphotransferase system of *E. coli*. Dotted arrows indicate that the phosphoryl group transfer from HPr can proceed to each of the EII^{mtl} subunits and that inter- and intradomain phosphoryl group transfer is possible as well.

ning α -helices and two large cytoplasmic loops (17), which is in accordance with the recently resolved projection map of the C domain (7). The location of the loops, however, is not known. A photocross-linking approach to identify the B/C domain interface suggested that the end of the first cytoplasmic C domain loop is located in the vicinity of Cys³⁸⁴ in the B domain.⁴ Here we describe the generation of disulfide bridges of cysteines within and between B and C domains. This work demonstrates that the first cytoplasmic loop of the C domain is in close proximity to the B domain active site and near the dimer interface.

EXPERIMENTAL PROCEDURES

Chemicals—Decyl-polyethylene glycol (dPEG) was synthesized by B. Kwant (Kwant High Vacuum Oil Recycling and Synthesis, Bedum, The Netherlands). Bovine pancreas L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was from Sigma and endoproteinase Glu-C of *Staphylococcus aureus* V8 was from Fluka. The bimalimides *o*-PDM and *p*-PDM were from Aldrich and BMH was obtained from Pierce. EI and HPr were purified as described previously (18, 19). All chemicals used were analytical grade.

Construction of Plasmids for Expression of SSCS, SSCS-S124C, and IICHis-S124C—Site-directed mutagenesis was performed with the Stratagene Quickchange mutagenesis kit. His-tagged EII^{mtl} with cysteines at positions 110, 320, and 571 replaced by serine (SSCS) was constructed in pMaHisMtlAPr, which carries the gene for the N-terminal His-tagged EII^{mtl}.⁵ Subsequently, SSCS-S124C was generated by replacing Ser¹²⁴ with a cysteine. IICHis-CL was generated by replacing Cys¹¹⁰ and Cys³²⁰ by serine in pMaMtlIICHis, which carries the gene for the C domain with a C-terminal His-tag (16). IICHis-S124C was then generated by replacing Ser¹²⁴ by a cysteine as described above. Mutations were identified by introducing silent restriction sites and confirmed by DNA sequence analysis.

Generation of ISO Vesicles and Purification of SSCS, SSCS-S124C, and IICHis-S124C—Growth of *E. coli* LGS322 (F[−] *thi*-1, *hisG1*, *argG6*, *metB1*, *tonA2*, *supE44*, *rpsL104*, *lacY1*, *galT6*, *gatR49*, *gatR50*, Δ (*mtlA*'p), *mtlD*[−], Δ (*gutR*'MDBA-*recA*)) carrying the various plasmids, and procedures to overexpress the mutant proteins were identical to those described for wild-type EII^{mtl} (20). Inside-out (ISO) membrane vesicles containing the mutant proteins were obtained as described (21). SSCS and SSCS-S124C were purified by Ni-NTA-agarose affinity chromatography as described for 6HEII^{mtl} (14), except that dPEG was used as the detergent. IICHis-CL and IICHis-S124C were purified as described (16).

EII^{mtl} Concentration Determination and Mannitol Binding and Phosphorylation Activities—The dissociation constant for mannitol binding and EII or IIC concentration in vesicles was determined by flow dialysis

after solubilization with 0.25% dPEG (22) with some modifications as will be described elsewhere.⁶ The mannitol phosphorylation activity was determined at 1 mM mannitol (23). The concentration of purified SSCS and SSCS-S124C was determined with the pyruvate burst assay, which determines the amount of PTS phosphorylation sites (24). The activities of IICHis-CL and IICHis-S124 were determined after formation of a heterodimer with EII^{mtl}-G196D, using 33 μ M mannitol as described (25).

Disulfide Cross-linking—A solution of $\sim 6 \mu$ M enzyme in ISO vesicles or 1 μ M purified protein was brought to a final concentration of 5 mM DTT and 20 mM EDTA from stocks of 0.1 and 0.5 M, respectively. If appropriate, 90 nM EI was added. After 15 min of incubation at 30 °C, DTT and EDTA were removed on a Bio Micro-spin 6 column (Bio-Rad), equilibrated with 50 mM NaP_i, pH 7.5, 0.1 mM EDTA, with (purified enzyme) or without (ISO vesicles) 0.25% dPEG. The buffer was deaerated with helium before addition of the detergent. Subsequently, HPr, MgCl₂, dPEG, and PEP were added to phosphorylate the purified enzyme, provided EI was present; these additions increased the final volume by 50%. The same mixture without PEP was used to represent conditions in which the enzyme was not phosphorylated. The final concentrations of the components were 2 μ M HPr, 5 mM PEP, and 5 mM MgCl₂. After 5 min at 30 °C, disulfide bridge formation was initiated by oxidation with 0.1 volume of 3 mM Cu(II)-(1,10-phenanthroline)₃ (CuPhe), followed by incubation at 30 °C for 30 min. The reaction was quenched by the addition of 65 mM EDTA from a stock of 0.5 M NaEDTA, pH 8.

Partial Digestion and Reduction—The protein was partially digested with 20 μ g of trypsin or 100 μ g of endoproteinase Glu-C/ml of reaction mixture for 1 h at room temperature. The digestion of the vesicles with endoproteinase Glu-C was done in the presence or absence of 0.4% dPEG. The digestion was stopped by the addition of SDS-PAGE denaturation buffer without β -mercaptoethanol. If appropriate, reduction was accomplished by the addition of 10 mM DTT after digestion.

Heterodimer Formation—Heterodimers between 3 μ M SSCS and 1 μ M IICHis-S124C or 0.2 μ M SSCS-S124C and 3 μ M IICHis-CL were formed by mixing purified proteins, followed by an incubation at 30 °C for 30 min. To promote heterodimer formation between SSCS-S124C and IICHis-CL, 170 mM Na₃PO₄ was added from a 1 M stock solution at pH 7.6. This lowers the cloudpoint of the detergent (dPEG), in which the protein is solubilized. This treatment results in dissociation of the initially homodimeric enzymes and thereby facilitates the mixing of the species (26). Subsequently, the heterodimers were treated as described above for the disulfide cross-linking procedure except that EI, HPr, MgCl₂, and PEP were omitted from the mixture.

Cross-linking with Dimalimides of Varying Length—1 μ M Purified IICHis-S124C was reduced and demetalated as described under "Disulfide Cross-linking." Cross-linking was initiated by adding to the protein, in 50 mM NaP_i, pH 7.5, 0.1 mM EDTA plus 0.25% dPEG, 5 μ M *o*-PDM, *p*-PDM, or BMH from a 10 times concentrated stock solution in *N,N*-dimethylformamide. The reaction was stopped with 10 mM DTT after incubation at 30 °C for 30 min.

SDS-PAGE Analysis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was done with 10% acrylamide gels as described (27). A denaturation buffer without β -mercaptoethanol was used. The samples were not boiled in denaturation buffer, because this leads to aggregation. The proteins were visualized either by silver staining (28) or by immunodetection after the proteins were transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic blotting. Detection, using the Western LightTM chemiluminescence detection kit with CSP-DTM as the substrate, was performed as recommended by the manufacturer (Tropix Inc.). The first antibody was an anti-His antibody from Amersham Pharmacia Biotech or Roche Molecular Biochemicals, and the second antibody was an anti-mouse IgG alkaline phosphatase conjugate (Sigma).

MALDI-TOF Mass Spectrometry—A Coomassie-stained band containing the C domain, generated by tryptic digestion, was excised from an SDS-polyacrylamide gel and completely destained with 50 mM NH₄HCO₃ in 40% ethanol. Subsequently, the gel piece was washed three times with 200 μ l of 25 mM NH₄HCO₃ and cut into pieces of ~ 1 mm³. A 200- μ l volume of 50 mM β -mercaptoethanol in 25 mM NH₄HCO₃ was added and, after 2 h of mixing at room temperature, the peptides were extracted with two times 200 μ l of 60% acetonitrile, 0.1% trifluoroacetic acid by 5 min of sonication in a bath sonicator. The β -mercaptoethanol solution and the extracts were pooled and dried in a Speed-Vac. The last traces of ammonium bicarbonate were removed by adding

⁴ B. A. van Montfort, R. H. Durkens, B. Canas, J. Godorac-Zimmerman, and G. T. Robillard, unpublished results.

⁵ E. Vos, personal communication.

⁶ E. Vos, J. Broos, and B. Poolman, unpublished data.

10 μ l of 1% trifluoroacetic acid and subsequent drying in the SpeedVac. The dried samples were dissolved in 5 μ l of 50% acetonitrile, 0.1% trifluoroacetic acid and sonicated for 5 min. Aliquots of 0.75 μ l were applied onto the MALDI target and allowed to air dry. Subsequently, 0.75 μ l of 10 mg/ml α -cyano-4-hydroxysuccinamic acid in 50% acetonitrile, 0.1% (v/v) trifluoroacetic acid was applied to the dried samples, which was then allowed to dry again. MALDI mass spectra were recorded with a Micromass Tofspec E MALDI time-of-flight mass spectrometer operated in reflectron mode. Spectra were calibrated externally.

RESULTS

Generation and Characterization of SSCS, SSCS-S124C, IICHis-CL, and IICHis-S124C—Two His-tagged EII^{mtl} mutants were constructed. SSCS is a single cysteine enzyme with only Cys³⁸⁴ in the B domain. SSCS-S124C contains Cys¹²⁴ in the C domain in addition to Cys³⁸⁴. The phenotype of *E. coli* LGS322 expressing these mutants was analyzed on MacConkey agar plates with 1% D-mannitol. Both strains formed purple-red colonies, indicating that the mutants transport and ferment mannitol. Inside-out (ISO) membrane vesicles from LGS322 cells expressing both mutants were solubilized with 0.25% dPEG and analyzed for mannitol binding and phosphorylation activities. The dissociation constants for mannitol were 25 and 45 nM and the turnover values for phosphorylation of mannitol of these mutants were 5200 and 4900 min⁻¹ for SSCS and SSCS-S124C, respectively. The turnover values for mannitol phosphorylation of purified SSCS and SSCS-S124C were 3200 and 2700 per min, respectively. These activities are similar to that of wild-type EII^{mtl} (22, 29). ISO membrane vesicles bearing IICHis-CL or IICHis-S124C, solubilized with 0.25% dPEG, and analyzed for mannitol binding, displayed dissociation constants for mannitol of 32 and 70 nM, respectively. These dissociation constants are similar to previously determined values of the wild-type C domain generated by tryptic digestion of the complete protein (22) or of the separately expressed IIC (8). In addition, IICHis-CL and IICHis-S124C could both complement the mannitol-binding defective EII^{mtl}-G196D for phosphorylation activity in an *in vitro* assay, a result similar to that described for IIC (8). Overall, these kinetic data clearly indicate that SSCS and SSCS-S124C are fully functional enzymes and that both mutant C domains can bind mannitol and form functional heterodimers with EII^{mtl}.

Disulfide Bridge Formation in ISO Vesicles and Purified Enzyme—Fig. 2A shows the results of CuPhe-induced oxidation of SSCS and SSCS-S124C in ISO membrane vesicles, as visualized by immunoblotting with an antibody raised against the N-terminal His-tag. The untreated proteins migrated as a major band at ~60 kDa and are indicated as EII. The band at 36 kDa is a degradation product, whereas the one at 116 kDa (band 1) is most likely the dimer. This dimer has been observed previously upon extraction of the enzyme from the membrane (3) and is most likely not held together by a disulfide bond, since it is resistant to reduction with DTT. The amount of this band 1 is the same in all lanes. The oxidation by CuPhe of both mutants resulted in the appearance of a higher molecular weight band (band 2). The molecular mass was ~200 kDa but varied depending on the concentration of acrylamide that was used (not shown). The formation of this band was almost completely reversed by reduction of the sample with DTT, suggesting that it is stabilized by a disulfide bridge. The reduction with DTT is not complete in the particular sample in lane 5. However, in lane 11 of Fig. 2A, lane 1 of Fig. 2B, and in duplicate experiments complete reduction was observed. Both high molecular weight bands (1 and 2) have been observed previously and both were denoted as dimeric species (4, 5, 30). Also in this paper we refer to band 2 as a dimeric species, but we cannot fully exclude the possibility that it represents another oligo-

meric state as will be discussed below. Since Cys³⁸⁴ is the only candidate for disulfide formation in SSCS, the enzyme is thus capable of forming a disulfide between the two Cys³⁸⁴ residues. This is an important observation, because the dimer contacts are between the C domains (8).

Partial digestion of EII^{mtl} with endoproteinase Glu-C generated a band at 33 kDa, which corresponds to the C domain without the A and B domains. Endoproteinase Glu-C instead of trypsin was used, because it did not cleave off the N-terminal His-tag within the 1-h incubation period. The cleavage pattern of digested SSCS was the same irrespective of whether the disulfide was formed or not. In addition, the cleavage pattern was unchanged upon reduction. This shows that the disulfide bridge resides in the domains that were degraded, which is consistent with the location of Cys³⁸⁴ in the B domain. However, if the same oxidation and digestion procedure was followed with oxidized SSCS-S124C, a new band at 50 kDa appeared (Fig. 2A, indicated by the arrow), irrespective of whether the digestion was done in the presence or absence of the detergent dPEG. This band disappeared upon reduction of the sample with DTT. This excludes the possibility that the protein was only partially cleaved under the oxidizing conditions. Based on its size, this band is probably the result of a disulfide bridge between Cys¹²⁴ of both monomers, indicating that residue 124 is at the dimer interface. Very vaguely, some other products, which could include a disulfide bridge between Cys³⁸⁴ and Cys¹²⁴ (see below) might be visible as well.

To further examine the nature of the Cys¹²⁴-Cys¹²⁴ disulfide bridge, the CuPhe-induced oxidation was repeated with purified SSCS-S124C. Fig. 2B shows the result of this experiment, visualized by immunoblotting. The same observations were made when the gel was silver-stained (not shown). The oxidation of purified SSCS-S124C also yields the reducible dimer band 2. Almost no band 1 was observed after purification, which probably indicates that the affinity between two monomers is decreased. Upon endoproteinase Glu-C digestion, the 50-kDa fragment was not observed. Instead, a His-tagged fragment of 42 kDa was visible in addition to the 33-kDa C domain band. This 42-kDa band disappeared upon reduction of the sample with DTT (compare lanes 3 and 4). Endoproteinase Glu-C digestion of the B domain will generate a 7.9-kDa fragment containing Cys³⁸⁴. The size of the 42-kDa band thus suggests that a disulfide bond is formed between the 33-kDa C domain harboring Cys¹²⁴ and the 7.9-kDa B domain fragment with Cys³⁸⁴. In conclusion, the data in Fig. 2, A and B, point to the formation of two different disulfides, one intersubunit Cys¹²⁴-Cys¹²⁴ and one interdomain Cys¹²⁴-Cys³⁸⁴. Further evidence for both disulfides will be supplied in the following sections.

Requirements for B/C Domain Disulfide Bridge Formation—To elucidate the composition of the disulfide bridges, the CuPhe-induced oxidation was performed with purified SSCS-S124C and SSCS (Fig. 3). The two higher molecular weight bands (1 and 2) were observed again with both proteins (lanes 2 and 14). These bands were not present upon reduction with DTT and were much less intense or absent upon phosphorylation of the protein at His⁵⁵⁴ and Cys³⁸⁴, confirming that these bands, at least in SSCS, arise from a disulfide bridge between the two Cys³⁸⁴ residues in the dimeric complex. Instead of endoproteinase Glu-C, trypsin was used to define the cross-links. Trypsin first cleaves in the linker between the C and B domain and, subsequently, digests the A and B domain completely but leaves the C domain intact except for the N-terminal His-tag (22, 31). The proteolytically generated C domain can be observed on SDS-PAGE at 30 kDa, which is somewhat smaller than the endoproteinase Glu-C-generated C domain.

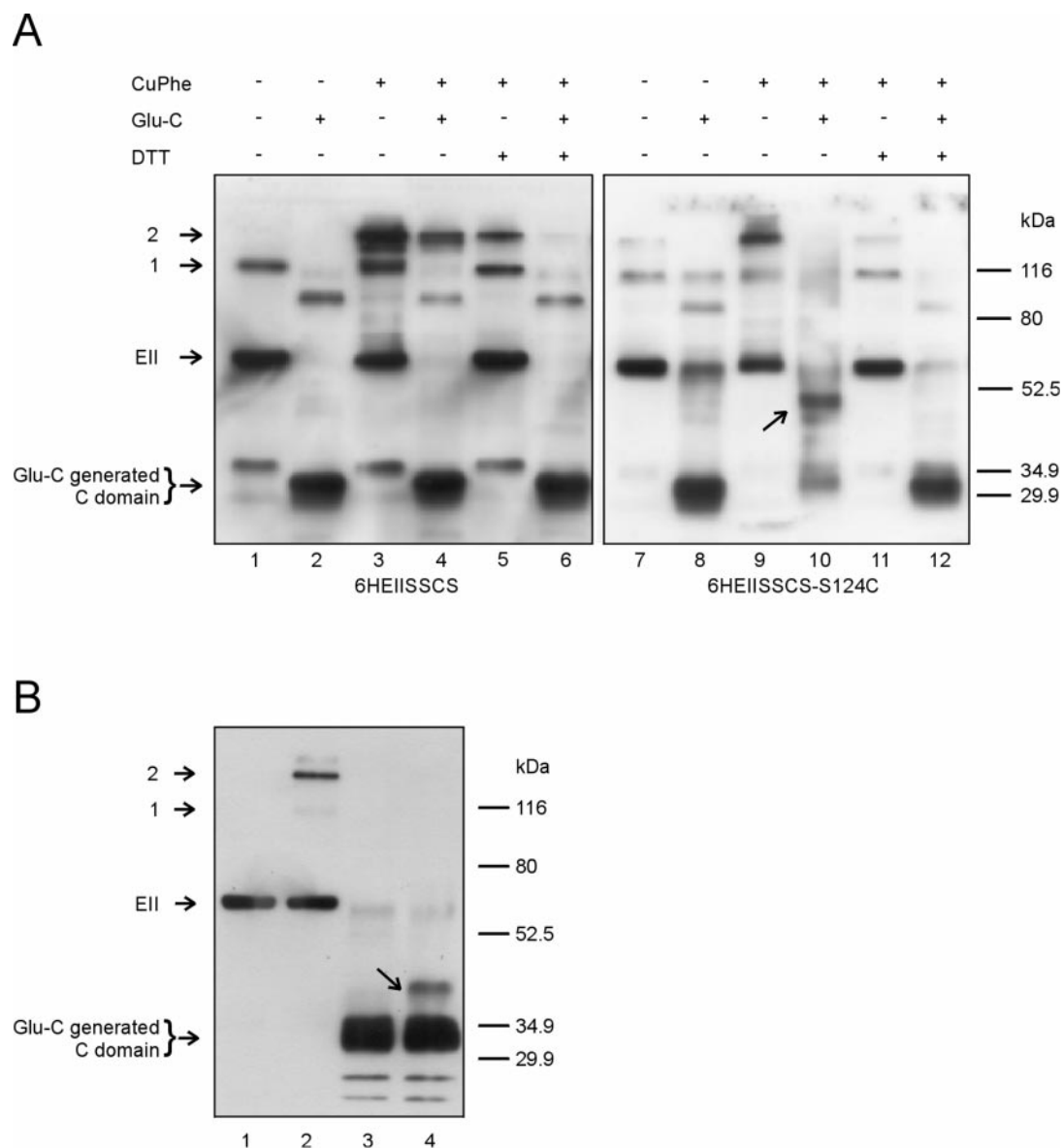


FIG. 2. Immunoblot analysis of CuPhe-induced disulfide bridge formation in SSCS and SSCS-S124C in inside-out membrane vesicles (A) and in purified SSCS-S124C (B). A, after oxidation, the samples were left untreated, digested with 100 μ g/ml endoproteinase Glu-C, reduced with 10 mM DTT, or digested and subsequently reduced as described under "Experimental Procedures." The conditions are depicted in the figure. The *left panel* (lanes 1–6) depicts the data with SSCS and the *right panel* (lanes 7–12) that of SSCS-S124C. The *arrow* indicates the location of the 50-kDa band, which was formed in SSCS-S124C only. The bands denoted 1 and 2 represent different conformers of EII (see text). Not shown are both mutants with added DTT but not oxidized, because these samples were identical to those that were DTT-reduced after CuPhe oxidation. B, approximately 1 μ M SSCS-S124C was oxidized with CuPhe and, subsequently, reduced with DTT (lane 1), left untreated (lane 2), endoproteinase Glu-C digested and, subsequently, reduced with DTT (lane 3) or endoproteinase Glu-C digested (lane 4). The *arrow* indicates the 42-kDa band. Approximately 0.5 μ g (A) or 0.08 μ g (B) of EII^{mtl} was loaded per lane.

This is consistent with the removal of the His-tag and a different cleavage site in the linker between the B and C domain. The tryptic B domain fragment with Cys³⁸⁴ has a calculated mass of ~1.9 kDa. Tryptic digestion of CuPhe-oxidized SSCS-S124C also showed an ~30-kDa band, which was significantly broadened upwards when the sample was not treated with DTT (compare lanes 6 and 7). This suggests that the broadened 30 kDa comprises the C domain plus the 1.9-kDa fragment of the B domain, analogous with the 42-kDa band in Fig. 2B. Consistent with this conclusion are the following observations: (i) the broadening is not observed with SSCS (Fig. 3: compare lanes 17 and 18); (ii) phosphorylation prior to oxidation prevented the occurrence of the broadening (compare lanes 6 and 8); and (iii) the broadening was not observed in wild-type EII^{mtl} or in mutants of SSCS with cysteines at positions 158 or 199 (not shown). A tryptic-generated C domain of SSCS-S124C, not

treated with CuPhe, also led to some broadening, which is probably due to spontaneous oxidation (compare lanes 5 and 6). Another point to note is that the addition of 100 μ M mannitol did not have an effect on the occurrence of the broadening (lane 9). Finally, dimeric C domain was not observed in the tryptic digest of oxidized SSCS-S124C.

Taken together, the broadening must be the result of a disulfide bridge between Cys³⁸⁴ and Cys¹²⁴. To exclude the possibility that this disulfide bridge is an aspecific reaction between two accessible cysteines, another control experiment was performed (Fig. 3, lanes 11 and 12). With the sample in lane 11, oxidation was carried out after trypsin treatment, whereas in lane 12 it was carried out before trypsinolysis. Clearly, the broadening of the C domain band is no longer observed when the trypsin digestion preceded the oxidation. Thus, Cys¹²⁴ is not capable of reacting with just any cysteine-

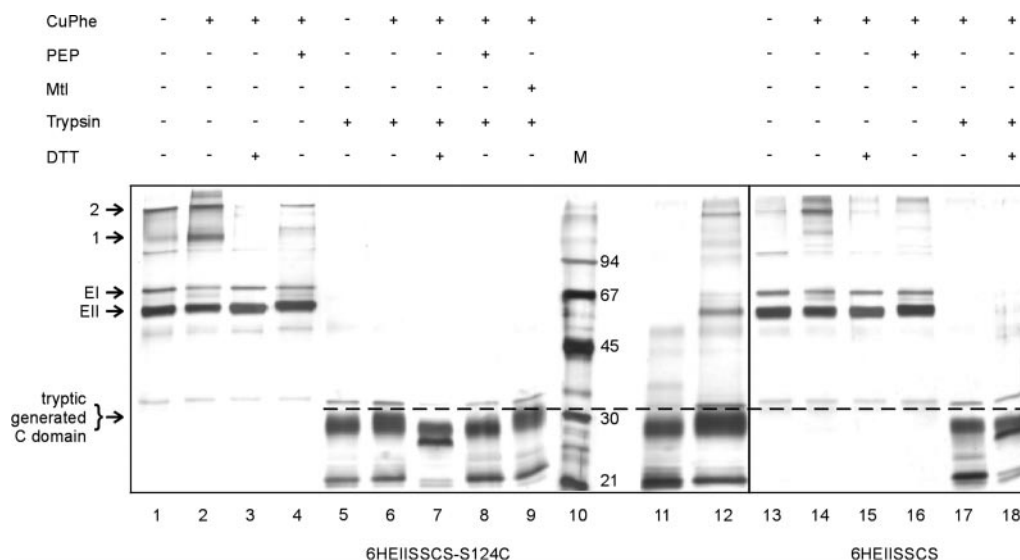


FIG. 3. SDS-PAGE analysis of the effect of phosphorylation and mannitol binding on CuPhe-induced cross-linking of purified SSCS-S124C and SSCS. The left panel (lanes 1–12) depicts the data with SSCS-S124C and the right panel (lanes 13–18) that of SSCS. The addition of PEP and EI plus HPr is indicated by “PEP.” After oxidation, the samples were left untreated, digested with 20 μ g/ml trypsin, reduced with 10 mM DTT, or digested and subsequently reduced as described under “Experimental Procedures.” Conditions are depicted in the figure. The dashed line makes the difference in height between oxidized and reduced IIC in SSCS-S124C more clearly visible. The bands denoted 1 and 2 both represent dimeric EII. Lane 10 contains the molecular weight marker. Lanes 11 and 12 show additional control experiments with SSCS-S124C. Lane 11, trypsin was added to the reduced enzyme and, after 1 h at 30 °C, oxidation was induced, which after 30 min was quenched with EDTA. Lane 12, the reduced protein was left at 30 °C for 60 min, after which CuPhe was added. After 30 min, EDTA and trypsin were added and incubated for another 60 min at room temperature. Approximately 0.1 μ g of EII^{mtl} was loaded per lane. In addition, shorter oxidation times (10 min instead of 30) yields the same broadening.

containing peptide, present at the same concentration.

MALDI-TOF Mass Analysis—To demonstrate that the fragment, which caused the broadening of the tryptic-generated C domain, originated from the B domain, the protein was excised from the SDS-polyacrylamide gel, reduced with β -mercaptoethanol to cleave the disulfide bond between the C domain and the B domain peptide, the peptides were extracted with organic solvents, and the extract was analyzed with MALDI-TOF MS. Fig. 4 shows the mass spectrum of the extracted peptides. The spectrum only contained 4 peaks, all of which could be assigned to tryptic B domain fragments that contain Cys³⁸⁴. The peak at m/z 1879.05 is the fully cleaved peptide with residues 380–399 (expected m/z 1878.92), and the peak at m/z 2007.05 represents the partially cleaved fragments of residues 379–399 and/or 380–400 (expected m/z 2007.02). This partial cleavage is due to the presence of the RK and RKK sequences at the N and C terminus of these peptides, respectively, that cannot be fully cleaved by trypsin. The peaks at m/z 1954.79 and 2083.05 represent the same peptides but with a β -mercaptoethanol adduct, which gives a mass increase of 76 Da. If the same procedure was applied to the C domain band that was reduced prior to SDS-PAGE and excised from the gel following the same procedure no peptides were observed.

The B/C Disulfide Bridge Can Cross the Dimer Interface—To this point, we have provided evidence for a B/C interdomain cross-link between Cys³⁸⁴ and Cys¹²⁴ in purified SSCS-S124C, and a cross-link between the Cys¹²⁴ of both monomers in the enzyme in ISO membrane vesicles. In addition, the data on SSCS provide unequivocal evidence for a disulfide across the dimer interface between the Cys³⁸⁴ residues on each B domain. To establish whether the disulfide between Cys³⁸⁴ in the B domain and Cys¹²⁴ in the C domain could be formed between different subunits, heterodimers consisting of IICHIS-S124C and SSCS were subjected to CuPhe-induced oxidation. Each subunit in a heterodimer provides only one of the cysteines that participate in the disulfide bridge. Fig. 5A (lane 2) shows that when a 3:1 mixture of SSCS and IICHIS-S124C was oxidized, the same homodimer as observed with purified SSCS alone

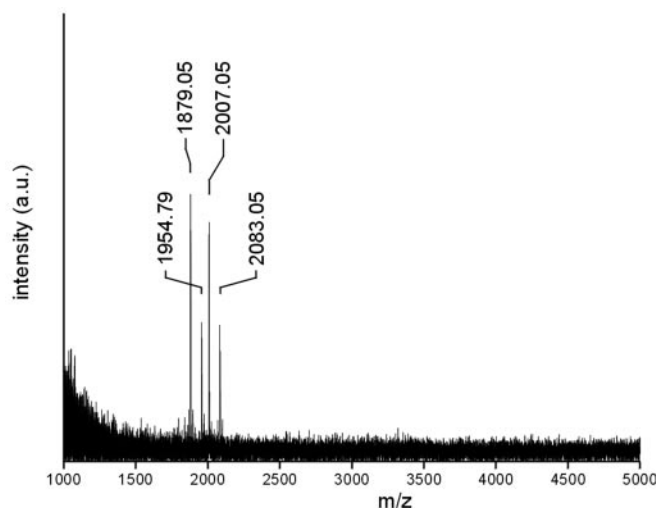


FIG. 4. MALDI-TOF mass spectrum of the B domain peptides extracted from a gel piece with the larger C domain. The peptides have been isolated as described under “Experimental Procedures.”

(Fig. 5A, lane 4, and Figs. 2 and 3) was formed. In addition, a new band at ~90 kDa appeared, whereas the IICHIS-S124C band became less intense (lane 2). This 90-kDa band can only be the heterodimer between SSCS and IICHIS-S124C. After reduction, both the SSCS dimer and the heterodimer disappeared. This shows that the B/C domain disulfide bridge can cross the dimer interface.

The B/C Disulfide Bridge Can Be Formed Intramolecularly—Next, we addressed the question whether or not the disulfide bridge between Cys³⁸⁴ and Cys¹²⁴ can also be formed within one monomer. For this purpose, heterodimers of SSCS-S124C and a cysteine-less IICHIS (IICHIS-CL) were formed. It is crucial for this experiment that all the SSCS-S124C monomers are forming a heterodimer with a IICHIS-CL monomer, such that only the intrasubunit disulfide bridge is possible. Therefore, an ~15-fold excess IICHIS-CL was added and the het-

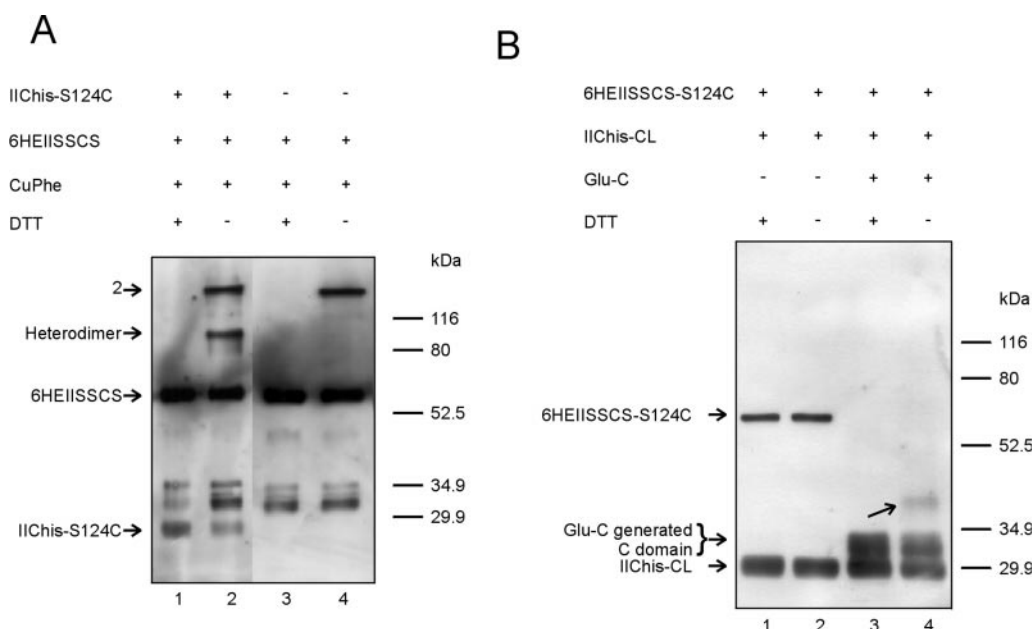


FIG. 5. Heterodimer experiments to determine whether the Cys³⁸⁴-Cys¹²⁴ disulfide can be formed intra- and intermolecularly. *A*, immunoblot of CuPhe-induced oxidation of homo- and heterodimers of purified SSCS (3 μ M) and IICHIS-S124C (1 μ M). After CuPhe-induced oxidation, the samples were split and either reduced with 10 mM DTT or left untreated. Due to a poor blotting efficiency of the C domain alone, the apparent concentrations on the PVDF of EII^{mtl} and the C domain differ. *B*, immunoblot of CuPhe-induced oxidation of heterodimers formed between purified SSCS-S124C (0.2 μ M) and IICHIS-CL (3 μ M) in the presence of 170 mM Na₃PO₄, pH 7.6. After CuPhe-induced oxidation, the samples were reduced with DTT (lane 1), left untreated (lane 2), endoproteinase Glu-C digested and, subsequently, reduced with DTT (lane 3), or endoproteinase Glu-C digested (lane 4). The arrow indicates the 42-kDa band. Approximately 0.15 μ g (*A*) and 0.075 μ g (*B*) of EII^{mtl} was loaded per lane.

erodimer formation was facilitated by the addition of 170 mM Na₃PO₄, pH 7.6, which lowered the cloud point of the solution (see "Experimental Procedures"). Na₃PO₄ alone did not have an influence on the cross-linking behavior of SSCS-S124C (not shown). As can be seen in Fig. 5*B*, homodimers were no longer present upon oxidation with CuPhe, which is indicative of complete heterodimer formation (compare Fig. 2*B*, lane 2, and Fig. 5*B*, lane 2). Endoproteinase Glu-C digestion of the oxidized sample generated the 33-kDa C domain and the additional 42-kDa band (arrow in lane 4 of Fig. 5*B*); the latter is absent upon reduction of the sample with DTT. This experiment, therefore, proves that the B/C disulfide bridge can also be formed within one subunit.

Residue 124 Is Located Near the Dimer Interface—The CuPhe-induced cross-linking of SSCS-S124C in ISO membrane vesicles corresponds most likely to a disulfide bridge between Cys¹²⁴ of both monomers. The CuPhe-induced oxidation was repeated with purified IICHIS-S124C (Fig. 6). IICHIS-S124C migrates as a monomer with an apparent molecular mass of 28 kDa in the presence of DTT. Upon oxidation, a dimeric IICHIS-S124C with an apparent molecular mass of 50 kDa was observed. To confirm the close proximity of both Cys¹²⁴ residues in one dimer, reduced IICHIS-S124C was subjected to cross-linking with dimaleimides of different lengths, ranging from 7.7 to 15.1 Å. Fig. 6 shows that all three dimaleimides also yielded the formation of the 50-kDa dimer. These data indicate that the residue at position 124 is located at the dimer interface in purified IICHIS-S124C.

DISCUSSION

In this article we describe the generation of several disulfide bridges, indicative for close proximity, between two cysteines in the dimeric EII^{mtl}. In the enzyme in ISO membrane vesicles, intersubunit disulfide bridges between both Cys³⁸⁴ residues and between both Cys¹²⁴ residues are formed. Upon purification in the detergent dPEG, the intersubunit disulfide between both Cys³⁸⁴ residues is still formed. In addition, an interdo-

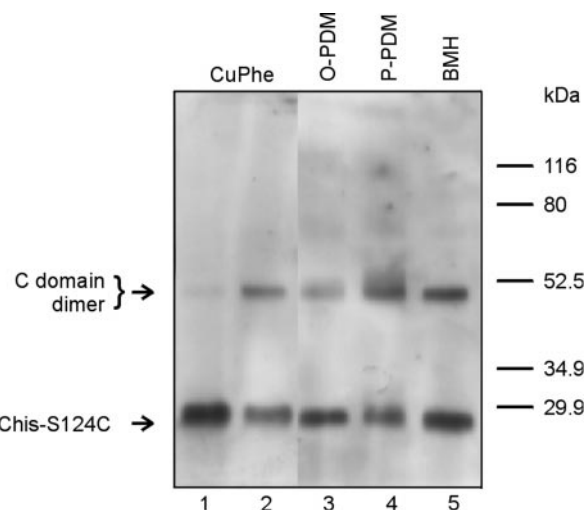


FIG. 6. Immunoblot of cross-linking experiments with purified IICHIS-S124C. IICHIS-S124C (1 μ M) was reduced and subjected to cross-linking by oxidation with CuPhe (lanes 1 and 2) or with *o*-PDM, *p*-PDM, and BMH, which can span 7.7, 12.0, and 15.1 Å, respectively. Lane 1 represents the sample in which DTT after CuPhe oxidation. Approximately 0.1 μ g of IICHIS-S124C was loaded per lane.

main cross-link between Cys³⁸⁴ and Cys¹²⁴ is observed, which either can be formed as an intrasubunit or an intersubunit disulfide. The intersubunit disulfide between both Cys¹²⁴ residues is not formed in purified EII, but it is formed in purified C domain harboring Cys¹²⁴. It is important to stress that both the detergent-solubilized EII and IIC retain full binding capacity, and the soluble EII retains full mannitol phosphorylation activity. The observations made with the detergent-solubilized enzymes thus reveal structural information of functionally relevant conformations of the protein.

To form a disulfide bond, the C β atoms of the cysteines have to come within 3.8–4.5 Å of each other (32). The two Cys³⁸⁴ and two Cys¹²⁴ residues are thus in very close proximity both at the

B/C domain interface and the dimer interface of EII^{mtl}. The multiplicity of possible disulfides is easy to understand in the light of the interdomain dynamics that are essential for the entire phosphorylation and transport cycle. The architecture and functioning of EII^{mtl} necessitates an interdomain flexibility in which the B domain interacts with various domains at different stages in the catalytic cycle. (i) The active sites of the A and B domains, each of which are proteins of ~15 kDa, must approach one another to transfer the phosphoryl group from His⁵⁵⁴ on the A domain to Cys³⁸⁴ on the B domain, then (ii) this same region of the B domain, containing a phosphorylated Cys³⁸⁴, must interact with the C domain to effect the conformational energy coupling which enables the translocation and subsequent phosphorylation of mannitol. (iii) The phosphoryl group can cross the dimer interface from the A domain of one monomer to the B domain of the other monomer (illustrated in Fig. 1) (33, 34) or from the B domain of one monomer to the mannitol bound by the C domain of another monomer (25, 35). It is, therefore, logical that the active site Cys³⁸⁴ in the B domain is in close proximity with different regions of the enzyme at different steps in the catalytic cycle and that, during the cross-linking process, these various domain interactions transiently occur and lead to the cross-links observed. Upon purification in detergent, a different pattern of the disulfide bridges is observed, which is additional evidence for this dynamic situation.

The observation of different higher aggregated forms of EII^{mtl}, all denoted as dimeric forms, is not new. Band 1 was observed upon extraction of the enzyme from the membrane, whereas band 2 appeared upon cross-linking via disulfides, dimaleimides, or lysine-specific cross-linkers (3–5, 30). Band 1 is insensitive to reduction suggesting that it is stabilized by noncovalent interactions rather than disulfides. In this regard, it is noteworthy that most observations of band 1 stem from measurements on protein extracted from the membrane but not purified. It is likely that its aggregation state is stabilized by lipids complexed to the protein. Band 2's position and, thus, its estimated mass varies with the degree of cross-linking in the polyacrylamide gel, but it is close to that of a tetramer. In a single-cysteine EII^{mtl}, the tetramer can be generated when a single disulfide bond cross-links two dimers whose subunits are held together by native-like interactions, as in the case of band 1. The tetramer could also arise from two disulfide-bonded dimers that come together to form a tetramer. The band in between 1 and 2 (lane 3, Fig. 2A) could represent the loss of a monomer from a tetramer, which is only possible in the inter-dimer cross-linked tetramer. A possible tetrameric state of the enzyme, however, does not lead to other conclusions for the close proximity of the cysteines at positions 384 and 124. In the future we will examine the possible tetrameric nature of EII^{mtl} in more detail with analytical ultracentrifugation as has been done for the lactose carrier of *Streptococcus thermophilus* (36).

As summarized in the Introduction, there is a lot of kinetic and thermodynamic evidence for conformational coupling at the B/C domain interface. However, there was no direct structural information about the location of this interface. Here, we present for the first time the location of at least part of the B/C domain interface, which is formed by the first cytoplasmic loop in the C domain and the region around the active-site cysteine in the B domain. The first evidence for the importance of this loop in the interaction with the B domain came from time-resolved fluorescence spectroscopy studies on a series of single-tryptophan containing mutants (13). Notably, a change in the time-resolved anisotropy of tryptophan 109 was observed upon phosphorylation of the B domain. The location around residue 124 is in a region which is conserved among mannitol-specific

EIIs of different origin (37). Close proximity of this region of the C domain and the B domain active site was already suggested by photocross-linking experiments.⁴ Recent kinetic data on a series of phenylalanine to tryptophan replacements in this first cytoplasmic loop suggest that residues 126 and 133 are critical for the phosphorylation of mannitol but not for mannitol binding. The data suggest that these residues are located at or near the dimer interface and involved in the B/C domain interaction,⁷ which is consistent with the data presented here.

The location of the interface in the first cytoplasmic loop is very interesting, because all previously described mutations that have an influence on the functioning of EII^{mtl} are located in the predicted second cytoplasmic loop. This is also the location of the GLXE motif (residues 254–257), which is highly conserved in all EII's and speculated to be involved in substrate binding (38). Replacement of Glu²⁵⁷ in this motif led to enzymes with no or only low affinity mannitol binding, depending on the substituent, as well as defective transport (39). Another region in the same loop is also important for activity. For instance, replacement of Gly¹⁹⁶ or His¹⁹⁵ led to enzymes, which exhibited no or low affinity binding for mannitol (25, 34, 40). Interestingly, a heterodimer of the inactive mutants E257A and H195A was significantly active in transport and phosphorylation of mannitol, suggesting that these residues are in close proximity (35). Also data on the glucose transporter of *E. coli* point to a role for the second cytoplasmic loop in substrate binding (41).

In conclusion, the data presented here suggests that residues 124 and 384 of both subunits can come within 5 Å of each other and are located at the B/C domain and dimer interface. An intriguing question is what happens at the B/C domain interface upon phosphorylation. Phosphorescence data of single tryptophan mutants in the C domain show that a conformational change takes place upon phosphorylation of Cys³⁸⁴, different from that upon mannitol binding (14). With the current pairs of cysteines, however, it is not possible to figure out what is happening precisely, because phosphorylation prevents Cys³⁸⁴ from forming a disulfide. In the future, we will screen several cysteine mutants in these regions of the protein to determine the exact borders of the B/C domain and dimer interface and changes therein upon mannitol binding and/or B domain phosphorylation. Eventually this will lead to a more detailed understanding of the energy coupling mechanism at the B/C domain interface in EII^{mtl}.

Acknowledgment—Jaap Broos is acknowledged for critical reading of the manuscript and useful discussions.

REFERENCES

- Postma, P. W., Lengeler, J. W., and Jacobson, G. R. (1993) *Microbiol. Rev.* **57**, 543–594.
- Robillard, G. T., and Broos, J. (1999) *Biochim. Biophys. Acta* **1422**, 73–104.
- Roossien, F. F., and Robillard, G. T. (1984) *Biochemistry* **23**, 5682–5685.
- Stephan, M. M., and Jacobson, G. R. (1986) *Biochemistry* **25**, 4046–4051.
- Pas, H. H., Ellory, J. C., and Robillard, G. T. (1987) *Biochemistry* **26**, 6689–6696.
- Lolkema, J. S., Kuiper, H., ten Hoeve Duurkens, R. H., and Robillard, G. T. (1993) *Biochemistry* **32**, 1396–1400.
- Koning, R. I., Keegstra, W., Oostergetel, G. T., Schuurman Wolters, G., Robillard, G. T., and Brissin, A. (1999) *J. Mol. Biol.* **287**, 845–851.
- Boer, H., ten Hoeve-Duurkens, R., Schuurman-Wolters, G. K., Dijkstra, A., and Robillard, G. T. (1994) *J. Biol. Chem.* **269**, 17863–17871.
- Lolkema, J. S., ten Hoeve Duurkens, R. H., Dijkstra, D. S., and Robillard, G. T. (1991) *Biochemistry* **30**, 6716–6721.
- Elferink, M. G., Driessen, A. J., and Robillard, G. T. (1990) *J. Bacteriol.* **172**, 7119–7125.
- Lolkema, J. S., Dijkstra, D. S., ten Hoeve Duurkens, R. H., and Robillard, G. T. (1991) *Biochemistry* **30**, 6721–6726.
- Boer, H., Tenhoeveduurkens, R. H., Lolkema, J. S., and Robillard, G. T. (1995) *Biochemistry* **34**, 3239–3247.
- Dijkstra, D. S., Broos, J., Visser, A. J. W. G., Vanhoek, A., and Robillard, G. T.

⁷ E. Vos, R. Ter Horst, G. T. Robillard, B. Poolman, and J. Broos, manuscript in preparation.

- (1997) *Biochemistry* **36**, 4860–4866
14. Broos, J., Strambini, G. B., Gonnelli, M., Vos, E. P., Koolhof, M., and Robillard, G. T. (2000) *Biochemistry* **39**, 10877–10883
15. Meijberg, W., Schuurman Wolters, G. K., and Robillard, G. T. (1998) *J. Biol. Chem.* **273**, 7949–7956
16. Meijberg, W., Schuurman Wolters, G. K., Boer, H., Scheek, R. M., and Robillard, G. T. (1998) *J. Biol. Chem.* **273**, 20785–20794
17. Sugiyama, J. E., Mahmoodian, S., and Jacobson, G. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 9603–9607
18. Robillard, G. T., Dooijewaard, G., and Lolkema, J. S. (1979) *Biochemistry* **18**, 2984–2989
19. van Dijk, A. A., de Lange, L., Bachovchin, W. W., and Robillard, G. T. (1990) *Biochemistry* **29**, 8164–8171
20. Robillard, G. T., Boer, H., van Weeghel, R. P., Wolters, G., and Dijkstra, A. (1993) *Biochemistry* **32**, 9553–9562
21. Broos, J., ter Veld, F., and Robillard, G. T. (1999) *Biochemistry* **38**, 9798–9803
22. Lolkema, J. S., Dijkstra, D. S., ten Hoeve Duurkens, R. H., and Robillard, G. T. (1990) *Biochemistry* **29**, 10659–10663
23. Robillard, G. T., and Blaauw, M. (1987) *Biochemistry* **26**, 5796–5803
24. Brouwer, M., Elferink, M. G., and Robillard, G. T. (1982) *Biochemistry* **21**, 82–88
25. Boer, H., Tenhoeveduurkens, R. H., and Robillard, G. T. (1996) *Biochemistry* **35**, 12901–12908
26. Broos, J., Hoeve Duurkens, R. T., and Robillard, G. T. (1998) *J. Biol. Chem.* **273**, 3865–3870
27. Laemmli, U. K. (1970) *Nature* **227**, 680–685
28. Blum, H., Beier, H., and Gross, H. J. (1987) *Electrophoresis* **8**, 93–99
29. Dijkstra, D. S., Broos, J., Lolkema, J. S., Enequist, H., Minke, W., and Robillard, G. T. (1996) *Biochemistry* **35**, 6628–6634
30. Roossien, F. F., van Es-Spiekman, W., and Robillard, G. T. (1986) *FEBS Lett.* **196**, 284–290
31. Stephan, M. M., and Jacobson, G. R. (1986) *Biochemistry* **25**, 8230–8234
32. Hazes, B., and Dijkstra, B. W. (1988) *Protein Eng.* **2**, 119–125
33. van Weeghel, R. P., van der Hoek, Y. Y., Pas, H. H., Elferink, M., Keck, W., and Robillard, G. T. (1991) *Biochemistry* **30**, 1768–1773
34. Weng, Q. P., Elder, J., and Jacobson, G. R. (1992) *J. Biol. Chem.* **267**, 19529–19535
35. Saraceni Richards, C. A., and Jacobson, G. R. (1997) *J. Bacteriol.* **179**, 5171–5177
36. Friesen, R. H., Knol, J., and Poolman, B. (2000) *J. Biol. Chem.* **275**, 33527–33535; Correction (2000) *J. Biol. Chem.* **275**, 40658
37. Henstra, S. A., Tolner, B., Duurkens, R. H. T., Konings, W. N., and Robillard, G. T. (1996) *J. Bacteriol.* **178**, 5586–5591
38. Lengeler, J. W., Titgemeyer, F., Vogler, A. P., and Wohrl, B. M. (1990) *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **326**, 489–504
39. Saraceni Richards, C. A., and Jacobson, G. R. (1997) *J. Bacteriol.* **179**, 1135–1142
40. Weng, Q. P., and Jacobson, G. R. (1993) *Biochemistry* **32**, 11211–11216
41. Ruijter, G. J., van Meurs, G., Verwey, M. A., Postma, P. W., and van Dam, K. (1992) *J. Bacteriol.* **174**, 2843–2850